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Consequences of frameshift mutations at the immunoglobulin heavy chain locus of the mouse

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From an IgM secreting hybridoma line we have isolated 16 spontaneous mutants that produce truncated IgM polypeptides. The size of the μ-mRNAs produced by these mutants is normal, but they express 3- to 100-fold less μ-mRNA and mutant μ-protein than the parental cell line. Nucleotide sequence analysis of cloned μ-genes and/or their mRNAs show frameshift mutations that generate in-phase chain termination codons. The extent of the reduction in μ-mRNA levels depends on the position of the nonsense codon within the gene.
Key words: immunoglobulin M/mRNA level/premature chain termination

Introduction

The immunoglobulin system offers a unique model to study eukaryotic gene structure and regulation of gene expression as well as the pathways of biosynthesis of proteins. It is most conveniently analysed by studying immunoglobulin (Ig) genes and their products in hybridoma cells with pre-defined specificity. A B cell produces large amounts of Ig. Ig synthesis is directed by only one of the chromosomes in diploid cells; and whether or not a cell produces Ig does not affect its viability. For these reasons the detection of Ig mutations is simpler than the detection of mutations in other proteins. Immunoglobulins are multichain proteins, which are assembled, glycosylated and either secreted or inserted into cell membranes, before they carry out defined biological activities. Numerous steps from gene activation to Ig production and final function of the protein are required. Hybridomas producing mutant proteins and the analysis of their genetic defects can help to elucidate the mechanisms of these various steps.

Ig genes are particularly susceptible to mutations (for a review, see Morrison and Scharff, 1981). Large effects on protein structure as determined by isoelectric focusing (Adetugbo *et al.*, 1977), size (Köhler *et al.*, 1982) or monoclonal antibody binding (Yelton and Scharff, 1982) have been shown. Deletions and insertions within Ig genes have been correlated with gross alterations of protein structure and expression (Adetugbo *et al.*, 1977; Köhler *et al.*, 1982; Baczynsky *et al.*, 1983; Hawley *et al.*, 1982). Frameshift mutations resulting in premature termination have been described (Adetugbo *et al.*, 1977) as well as mutants with altered subclass (Radbruch *et al.*, 1980).

We have isolated spontaneous mutants of IgM producing hybridomas. One large group contained deletions of DNA, which result in the loss of one, two or three μ-constant region domains (Köhler and Shulman, 1980; Köhler *et al.*, 1982; Shulman *et al.*, 1982). These deletion variants were used to map a panel of rat monoclonal antibodies to each of the four mouse μ-constant region domains (Leptin *et al.*, 1984). These antibodies, in turn, localized the defect of a second major fraction of spontaneous mutants, described here, to their carboxy-terminal ends.

In some of the mutants we determined the nature of the genetic events that lead to changes in protein size, synthesis, secretion and μ-mRNA levels.

Results

Origin of mutants

Spontaneous mutants were derived from the hybridoma cell line Sp6, which secretes an IgM specific for trinitrophenyl (TNP). The selection for the mutants has been described in detail (Köhler and Shulman, 1980). Briefly, the variants were selected by covalently coupling the hapten trinitrophenyl to the surface of Sp6 hybridoma cells, separating them from each

Table I. Origin of mutants

Selection no.	Name[a]	Parent	Fraction of Cμ-[a] DNA positive variants
1	*igm*10	Sp602	2/7
2	*igm*210*	Sp603	
3	*igm*662	Sp603.12.2	2/5
	*igm*320*	Sp603.12.2	
4	*igm*440*	Sp603.12.2.18	2/2
	*igm*1882	Sp603.12.2.18	
5	*igm*145	Sp603.12.2.18.1	5/6
6	*igm*482	Sp603.12.2.18.4	
7	*igm*710*	Sp603.12.2.18.5.A	8/8
	*igm*734	Sp603.12.2.18.5.A	
	*igm*7111	Sp603.12.2.18.5.A	
	*igm*725	Sp603.12.2.18.5.A	
8	*igm*810*	Sp603.12.2.18.5.B	
	*igm*820*	Sp603.12.2.18.5.B	
	*igm*8279	Sp603.12.2.18.5.B	
	*igm*8223	Sp603.12.2.18.5.B	
9	*igm*919*	Sp603.12.2.18.5.J	
	*igm*933	Sp603.12.2.18.5.J	
	*igm*927*	Sp603.12.2.18.5.J	

[a]The list contains previously described mutants (first six selections, Köhler *et al.*, 1982) and mutants additionally identified in selections 7–9, detected by screening for secretion and intracellular production of μ-proteins. Mutants marked by a star were derived from the screening (of all nine selections) for idiotype-negative variants (see Materials and methods). Presumptive re-isolates of known phenotypes resulting from this screening were not further analysed and are not listed, but were included in the initial screening for presence of constant region (Cμ)-DNA sequences. This explains the higher numbers in the last row as compared with the mutants marked by stars.

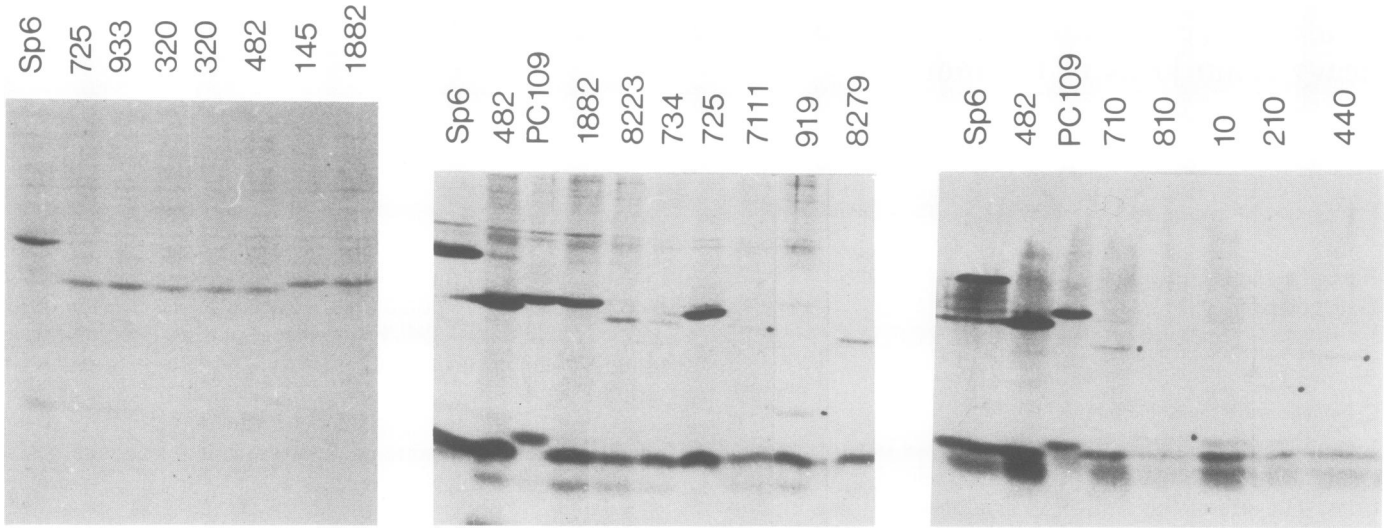

Fig. 1. SDS-PAGE analysis of mutant heavy chains. Cells were grown in the presence of tunicamycin and [^{14}C]leucine. Intra- and extracellular Ig was immunoprecipitated with rabbit anti-mouse Ig antiserum and reduced before analysis by SDS-PAGE. *igm*10 has no μ-chain but still expresses the non-specific κ-chain from its X63-Ag8 fusion parent and the Sp6-derived light chain with slightly faster electrophoretic mobility. PC109 is a C-terminal variant from PC700 an IgM, κ anti-phosphorylcholine secreting hybridoma line (Shulman *et al.*, 1982) shown here to co-migrate with *igm*1882. Weak bands are pointed out by dots. Sizes in kd of the mutants are summarized in Table II.

other in semi-solid medium, and incubating them in the presence of complement. Since the TNP-specific IgM preferentially binds to the TNP on the surface of the cell from which it was secreted, wild-type cells preferentially commit suicide. Thus cells secreting no IgM or altered IgM were enriched. After the above selection procedure was repeated twice, surviving cells were cloned as described (Köhler and Shulman, 1980). Table I summarizes nine independent selections and gives the names of the parental cell clones from which the variants were derived. Subclones of Sp6 are denoted by the addition of a numeral or a letter to the orignal name; sister reclones vary only in the last position, but not in length of the name.

The first three selected populations were screened for secretion of μ-chains, the later ones were also screened for intracellular production of μ-chains. Mutants found in the first six selections have been described (Köhler *et al.*, 1982). Carboxy-terminal variants of these selections (except *igm*10) and additional C-terminal variants identified in selections 7–9 are listed in Table I (names without a star). In addition, to reveal variants that produce no μ-chain, all nine selections were rescreened in a radioimmuno-inhibition assay for the absence of idiotypic determinants (see Materials and methods). Clones scored as idiotype negative might either have lost the chromosome bearing the μ-gene, as has been found to occur frequently in hybridoma cells (Hengartner *et al.*, 1978; Köhler, 1980), or they might have suffered a genetic defect in a structural or regulatory gene, leading to expression of no or a low amount of (altered) protein. To differentiate between these possibilies, the DNA of two to five such idiotype-negative clones from each selection was analysed for the presence of Cμ encoding sequences by Southern blots (data not shown). Surprisingly, absence of Cμ-sequences dominated only the first three selections (Table I, last row). In later selections, only one out of 16 variants had lost the Cμ-encoding sequences. Either re-isolates were picked or new ones (marked by a star for all selections) were identified. With the exception of *igm*320 all these mutants produce undetectable (*igm*820, *igm*927) or low amounts of μ-chains (*igm*210, *igm*440, *igm*710, *igm*810, *igm*919) (Figure 1). The variants *igm*820 and *igm*927 were found to have altered J-region DNA restriction patterns and were not included in this analysis. The variant *igm*10, which has no DNA hybridizing to either Jμ or Cμ-probes, was used as a control. All other variants had unaltered restriction fragments for their respective J_H or Cμ gene segment.

Protein analysis of the mutants

To estimate the size of the proteins produced, cells were treated with tunicamycin (TM) to obtain unglycosylated chains. Intra- and extra-cellular material was precipitated with rabbit anti-mouse IgM and subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions (Figure 1). Thus the wild-type (WT) μ-chain has an apparent mol. wt. of 60 kd (Table II). Ten mutants were found to produce μ-chains with mol. wts. from 47 kd to 49 kd (Table II), a size range that corresponds approximately to a μ-molecule which lacks one domain. All of these are unable to form pentamers but rather assemble into H_2L_2 monomers (Köhler *et al.*, 1982, and data not shown). The other six mutants produce small amounts of μ-chains of sizes from 28 kd to 45 kd (see Table II and Figure 1 pointed out by dots).

Mutants *igm*482, *igm*725, *igm*933 and *igm*320 secrete large amounts of H_2L_2 monomers, while *igm*145 and *igm*1882 secrete reduced amounts due to enhanced intracellular degradation (Sidman *et al.*, 1981) of H_2L_2 monomers. Similarly, mutants *igm*662, *igm*734, *igm*7111 and *igm*8223 also display very low levels of monomers, of which little is secreted.

To obtain information about which parts of the mutated μ-chains are missing, a series of experiments using monoclonal antibodies against distinct μ-domains (Lepin *et al.*, 1984) were performed (Table II). All lysates of the various lines were incubated in rabbit anti-mouse μ-coated polyvinyl chloride plates, and bound material was measured with radiolabeled monoclonal anti-mouse μ antibodies. Values with more than three times background (*igm*10, a variant of Sp6 producing only light chain), were considered positive. The smallest variants *igm*810 and *igm*919 only react with the anti-idiotypic reagent, indicating a correct association of a V_H-domain with

Table II. Characterisation of mutant μ chains using monoclonal antibodies

Mutant	% of μ-mRNA[a] relative to WT	Protein[b] +TM mol. wt. (kd)	20-5 [^{125}I]-[c] α-idiotype	R33-24[c] α-Cμ1	b7-6[c] α-Cμ2	M7/5[c] α-Cμ2	2911[c] α-Cμ3	1M 41[c] α-Cμ3	C2-23[c] α-Cμ4	M8/7.5[c] α-Cμ4
*igm*10	0	–	275	125	120	65	60	45	31	60
*igm*810	3	27	1600	110	150	40	150	90	87	190x
*igm*919	3	31	1700	100	90	68	150	66	67	170x
*igm*210	1	35	1700	600	90	50	98	51	48	107
*igm*440	1	41	610*	500	90	91	65	40	38	104
*igm*710	1	43	9200	2200	800	830	67	37	36	66
*igm*8279	30	45	5000	1900	500	260	90	46	44	108
*igm*8223	1	47	3600	1300	500	120*	60	30	69	58
*igm*7111	1	47	8200	2100	800	360	60	31	33	67
*igm*734	4	47	7500	2300	880	380	65	34	24	59
*igm*662	10	47	29 500	3800	510	1560	73	56	56	68
*igm*145	30	49	4800	2000	800	900	54	27	38	75
*igm*1882	30	49	16 500	3000	1370	1370	80	32	31	79
*igm*482	20	48	32 000	3400	2220	1100	1700	2700	55	78
*igm*320	10	48	+	+	+	+	+	+	–	–
*igm*725	30	48	33 000	3800	2220	900	1900	2400	40	66
*igm*933	30	48	+	+	+	+	+	+	–	–
Sp6	100	60	22 000	2100	1150	300	1500	1000	1100	1400

[a]Amount of specific μ-mRNA in the mutants relative to wild-type Sp6 is derived from dot blot analysis of μ-mRNA expression (Figure 2b).
[b]Protein size estimates, see Figure 1.
[c]All numbers indicated are the mean values of two measurements expressed in c.p.m. Values three times over background (*igm*10 a light chain only producer) are considered positive (below separation line), except numbers marked by *, which by inference may be considered positive. Numbers marked by x see Discussion.

V_χ, since neither of the V-regions alone react strongly with this antibody. Note that the direct binding assay using iodinated anti-idiotypic antibodies is at least 10 times more sensitive than the inhibition assay which was used to identify some of these mutants as idiotype-negative in the initial screening; this explains the discrepancy of the original screening and the binding assay shown here. The variants *igm*210 and *igm*440 also react with the anti-Cμ1 reagent, which also requires the presence of C_χ. The groups *igm*710 down to *igm*1882 (in Table II) bear V_H, Cμ1, and Cμ2 determinants. The 48-kd mutants (*igm*482 to *igm*933) display Vμ, Cμ1, Cμ2 and Cμ3 determinants. A good correlation between protein size and the presence of Cμ-domains is observed, indicating mutations at the carboxy terminus. The borderline positive binding of anti-Cμ4 antibody M8/7.5 to *igm*810 and *igm*919 will be discussed later (see Discussion).

Analysis of μ-specific mRNA

Figure 2a shows the Northern blot analysis of RNA from wild-type and the mutants. Unfractionated cytoplasmic RNA or poly(A)$^+$ RNA was run on a 1% agarose gel, transferred to a nitrocellulose filter and probed with pABμ-1 (Alt *et al.*, 1980), a cDNA probe specific for the μ-constant part. All mutants show the same 2.4-kb μ-specific RNA band as the Sp6 parental cell line, a fact which suggests that no large deletions have occurred (*igm*927, which has deleted the V-region, is shown as a control).

Even though all μ-mRNAs of the mutants were of wild-type size, we observed considerable differences in the amount of specific μ-mRNA expressed. To explore these findings further, we quantitated the amount of specific μ-mRNA by RNA dot blotting. The expression of *myc* mRNA, which should not be affected by μ-gene mutation, was used as internal control (data not shown). 20 μg of total, unfractionated cytoplasmic RNA was tested from each mutant and serially diluted with 3-fold steps. RNA was blotted to nitrocellulose filters and probed with pABμ-1 (Figure 2b).

Table II shows the relative amounts of mutant μ-mRNAs compared with wild-type Sp6 RNA expression. With the exception of *igm*8279, there is progressively less μ-mRNA the more upstream the mutational event has presumptively occurred, hence the more carboxy-terminal sequences the polypeptide chain should be missing.

DNA and RNA sequencing of eight mutants

DNAs of the mutants *igm*145 and *igm*482 were digested with the restriction enzyme *Eco*RI and cloned into the λ phages, λgtwes λB and λCh4A, respectively. The isolated μ-specific DNA clones were digested with restriction enzyme *Pst*I and subcloned into M13 mp8 phage. The subclones containing the Cμ3-Cμ4 segment were analyzed by dideoxy nucleotide sequencing (Sanger *et al.*, 1977).

*Igm*145 has a deletion of two consecutive bases at a position in the DNA sequence corresponding to amino acid position 426 (AA numbering according to Kawakami *et al.*, 1980) at the end of Cμ3 (Figures 3 and 4). This leads to a shift in the reading frame so that 19 missense amino acids are encoded before a stop codon at position 446 which is generated by splicing Cμ3 to Cμ4. Similarly the *igm*482 variant has a deletion of two consecutive bases at a position in the DNA sequence corresponding to amino acid position 439 (Figures 3 and 4), resulting in six missense amino acids encoded before a stop codon at position 446. The C-terminal ends of *igm*482 and *igm*145 are therefore identical.

To assess the mutational events in mutants *igm*734, *igm*662, *igm*1882, *igm*320, *igm*725 and *igm*933 and to confirm the structure of *igm*145 and *igm*482, we sequenced their μ-mRNAs in the third domain using a primer complementary to 17 bases

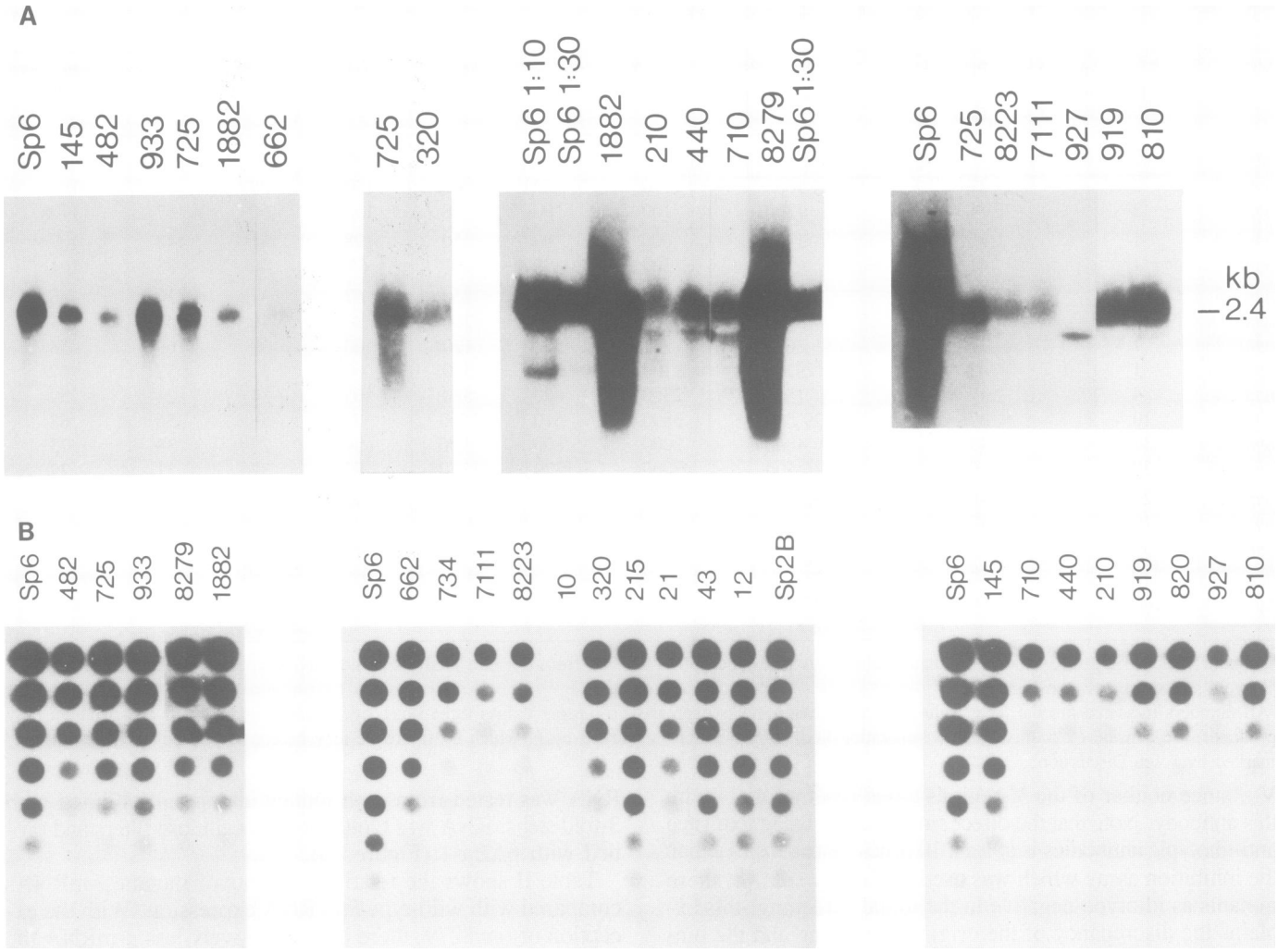

Fig. 2. Analysis of μ-specific RNA. RNA was prepared from the indicated cell lines, electrophoresed in 1% agarose (**a**) or blotted directly to nitrocellulose at serial dilutions of 1:3, starting with 20 μg of cytoplasmic RNA. (**b**) μ-specific RNA was revealed with pABμ-1, containing Cμ3 and Cμ4 specific sequences (Alt *et al.*, 1980). *igm*10, which makes no μ chain, serves as a negative control. *igm*215 is a low affinity variant; (Köhler and Shulman, 1980), *igm*21 a low synthesis variant, *igm*12 and *igm*43 are deletion variants (Köhler *et al.*, 1982), Sp2B is an unrelated IgM producing hybridoma (Köhler *et al.*, 1982), *igm*820 has a deletion or insertion 5′ of Cμ, *igm*927 misses the J-region and probably produces a sterile (Cμ−) transcript only (Kemp *et al.*, 1980).

at the beginning of the fourth domain (see Materials and methods). The results (shown for *igm*145, in Figure 3) confirmed the deletions observed by DNA sequencing for both *igm*145 and *igm*482 and proved that splicing of Cμ3 and Cμ4 indeed generated the expected opal stop codon.

In mutants *igm*662 and *igm*734, due to single base deletions at amino acid positions 394 and 396, respectively, 32 and 34 missense amino acids are encoded before an opal stop codon at position 428 (Figure 4). This leads to a protein which is 17 amino acids shorter than *igm*482 or *igm*145, in agreement with the protein size on SDS-PAGE and with monoclonal antibody mapping.

The μ-mRNA sequence of mutant *igm*1882 revealed a deletion event identical to that in *igm*145, thus explaining their similar secretion and degradation patterns and SDS-PAGE mobilities. Surprisingly *igm*725, *igm*933 and *igm*320 had identical deletions of two consecutive bases at a position in the RNA sequence corresponding to amino acid position 442 (see Figure 4). By this deletion an ochre stop codon is generated. Therefore, a protein is made which is three amino acids shorter than the one of clone *igm*482 which displays similar features. From sequencing data we expect the protein of *igm*482 to be more basic by one positive charge difference compared with the proteins of *igm*725, *igm*933 and *igm*320 respectively, and this expectation was confirmed by isoelectric focusing (IEF) (Figure 5).

Discussion

Loss of Ig production in hybridoma cells has been associated with loss of the respective chromosomes (Hengartner *et al.*, 1978; Köhler, 1980). Stabilization of Ig production is often observed after repeated recloning of hybridoma lines. We have also observed this effect in the Sp6 line. After four and six recloning steps, the lines Sp602, Sp603 and Sp603.12.2 were established (Table I). From 12 'non-producing' variants analysed (for their selection see Materials and methods), eight have lost Jμ- and Cμ-specific sequences in agreement with a presumptive chromosome loss. 'Non-producing' variants from later reclones of Sp6 (Sp603.12.2.18 and descendants) showed only one possible chromosome loss in 16 variants analysed (Table I, last row). Adding all published IgM mutants (Köhler

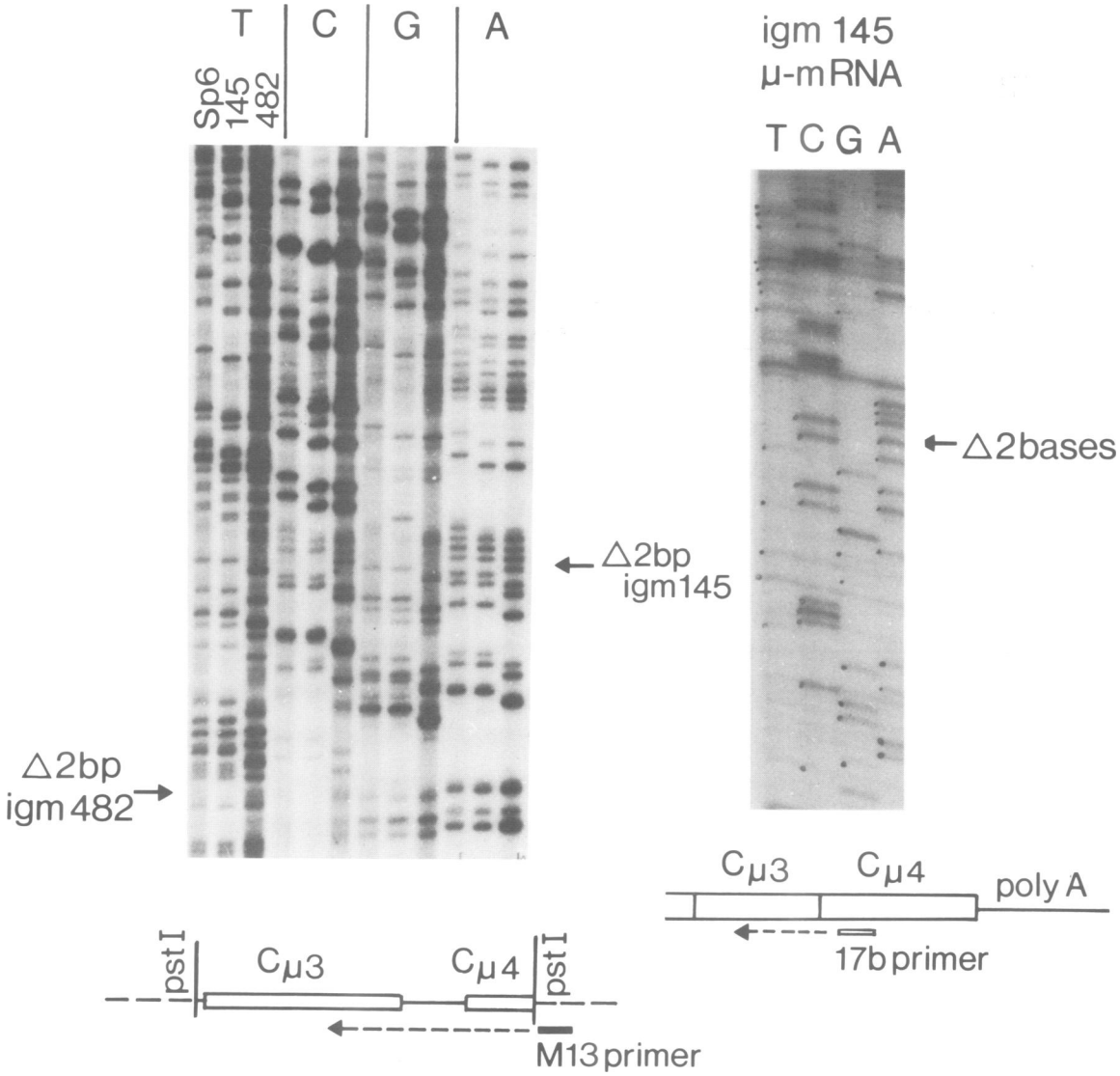

Fig. 3. Sequence analysis of mutants *igm*145 and *igm*482, with sequenced region and primers indicated below.

and Shulman, 1980; Köhler *et al.*, 1982; Shulman *et al.*, 1982; Baczynsky *et al.*, 1983) to the ones described here we find that of 43, 21 are consistent with being premature chain termination mutants, of which eight have been shown in this paper to be due to frameshift mutations. Early chain termination leads to low levels of mRNA (Table II) and to low Ig production and secretion. These variants represent the major group of variants detected in a stabilized hybridoma, in which chain loss due to chromosome loss is a less frequent event.

This raises the possibility that the high rates of chain-loss variants of $\sim 10^{-3} - 10^{-4}$ per cell and generation observed in myeloma lines (Coffino and Scharff, 1971; Cotton *et al.*, 1973) are predominantly caused by early termination mutations. If this is correct, the mutation rate of Ig genes in myelomas or hybridomas is orders of magnitudes higher than those observed for other genes ($< 10^{-7}$ per cell per generation) (Baumal *et al.*, 1973; Margulies *et al.*, 1976). One possible explanation could be an increased susceptibility of heavily transcribed genes for mutations. The fact that we observe regions that are particularly susceptible to mutations suggest mutational hot-spots. Such hot-spots have been defined in phages (Benzer, 1961; Okada *et al.*, 1972) and were also found in our analysis. Out of eight independent mutants, three (*igm*725, *igm*933 and *igm*320) and two (*igm*1882 and *igm*145) were identical.

In the gene encoding bacteriophage T4 lysozyme, it has been observed that stretches of either repeated bases or repeated base doublets increase the chances for misspairing and so render these stretches especially susceptible to frameshift mutations (Streisinger *et al.*, 1966). In the case of the mutants *igm*145 and *igm*1882 the 2-bp deletion occurs in a region where the doublet GT occurs three times. Mutants *igm*725, *igm*933 and *igm*320 have deleted 2 bp at an adenine-rich region (four adenines in a row). Similarly the mutations of *igm*482, *igm*734 and *igm*662 occur in regions with repeated adenines (three and four in a row, respectively). A similar mutant has been found in the γ_{2b},κ producing myeloma cell line MPC11, which has precisely deleted the third constant domain of the heavy chain by a 2-bp deletion (leading to an ochre stop codon) in an adenine-rich stretch immediately preceding the intron separating the second from the third constant domain of the γ2b gene (Kenter and Birshtein, 1979).

Several mutant proteins described here have already been analyzed for their secretion and processing properties and

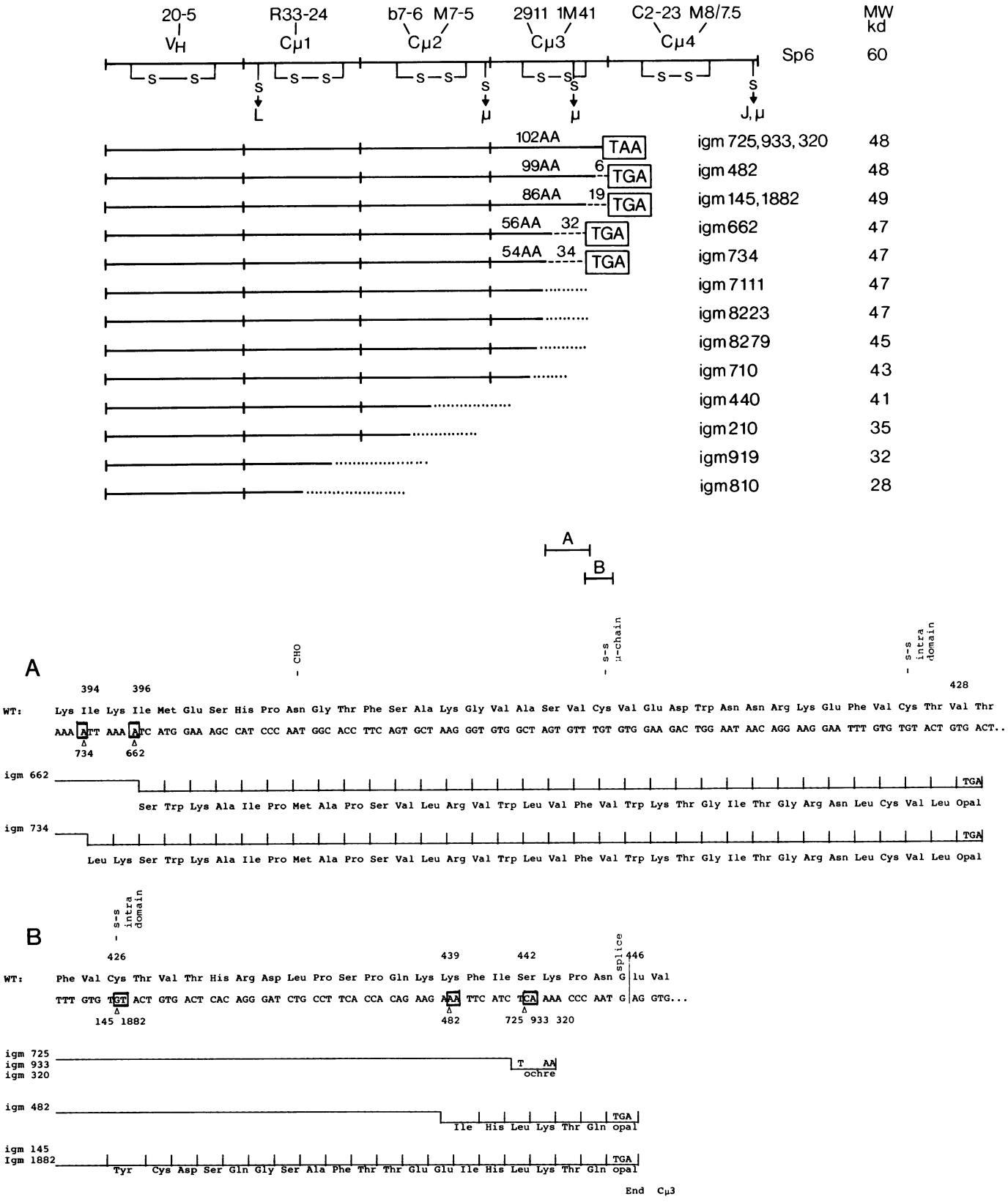

Fig. 4. Deletions in mouse μ-chains. The wild-type μ-protein Sp6 is drawn in its domain structure and disulphide bridges to the light (L) or heavy (μ), or J chain; and intradomain disulphide bridges are indicated. Size and structure of the unglycosylated mutant proteins are drawn, with out-of-frame amino acids (dashed lines and numbers) and stop codons indicated, in the case where the sequence of the DNA or RNA is known. In the other mutants, stops are deduced from protein size and the positions of mutation (assuming frameshifts) from the antibody binding studies. Names of monoclonal rat anti-mouse antibodies are written above the wild-type μ-chain to indicate where they presumably bind. They were selected from ~20 anti-mouse μ antibodies and define independent binding sites. The cell line 20-5 makes a mouse anti-idiotypic antibody reacting specifically with Sp6 IgM. Parts of the 3rd μ domain are enlarged (marked A + B) and their wild-type sequence printed out. The deletion points and out-of-frame amino acids of mutants *igm*662 and *igm*734, are shown in **A**, those of mutants *igm*725, *igm*933, *igm*320, *igm*482, *igm*145 and *igm1882 in* **B**.

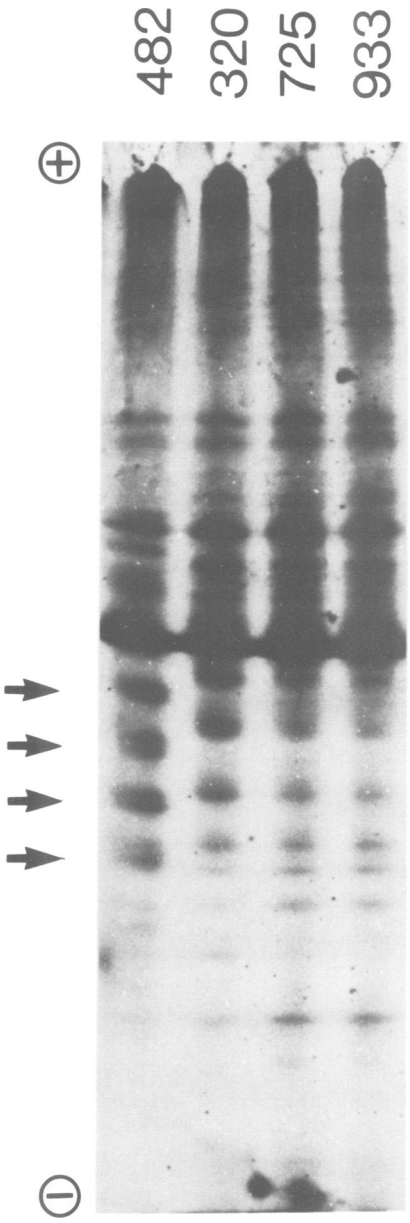

Fig. 5. Isoelectric focusing of mutant proteins. Cells were grown in [^{14}C]leucine-containing medium. Ig in supernatant was denatured and separated in a pH gradient from pH 5 (top) to pH 9 (bottom) according to their isoelectric points.

carbohydrate modification (Sidman *et al.*, 1981). The property of low secretion and fast degradation of *igm*145 and *igm*1882 compared with *igm*482 (Sidman *et al.*, 1981) seems to reside in the stretch of 13 amino acids which are out of frame in *igm*145. The shift of the cysteine residue 1 position downstream appears to destroy the structure of the whole third domain, probably by not allowing the intradomain disulfide bridge to be formed. This interpretation is supported by the failure of the two anti-Cμ3 antibodies to recognize this structure.

The unglycosylated form of the *igm*145 protein has been shown (Sidman *et al.*, 1981) to be more stable than the glycosylated form (half-life 175 min *versus* 15 min, respectively). It was proposed that due to frameshift a new glycosylation acceptor site is generated directing glycosylated protein into the lysosomal pathway which therefore accounts for the rapid degradation. Unfortunately the sequence analysis does not support this idea. There is no new N-linked CHO acceptor site created in the 19 out-of-frame amino acid stretch in mutant *igm*145.

Mutants *igm*810 and *igm*919 show a borderline positive binding with the anti-Cμ4 antibody M8/7.5, even though the mutation is presumably located in Cμ1. These clones might produce low amounts of protein, which could be the result of an unusual splicing of the V region to the Cμ3/4 region in addition to the correctly spliced mRNA. This possibility is suggested by the additional band that is sometimes observed in wild-type Sp6 mRNA (Figure 2a) and represents ~1% of the normal size (2.4 kb) mRNA. The size (1.2 kb) and the fact that we can detect it with either a V-region probe (data not shown) or a Cμ3+4 region probe would be in agreement with such an incorrect splicing.

Interestingly, the analysis of the μ-specific mRNA levels in the mutants show that frameshift mutations can interfere with the expression of the corresponding mRNA depending on their positions in the gene. This effect has been described in yeast (Losson and Lacroute, 1979). Nonsense mutations reduced the mRNA level without lowering its instantaneous rate of synthesis, thus affecting RNA stability. The strength of reduction depended on the position of the nonsense codon within the locus. Progressively higher specific mRNA levels were observed for mutations residing further away from the 5′ end of the gene. The effect was not linear, as mutants in the first half of the gene had a very low level of specific mRNA. Our mutants display the same properties. Mutants *igm*210, *igm*440, *igm*710, *igm*8223 and *igm*7111 have very low levels of RNA compared with mutants in the C-terminal part of the third domain, which have almost wild-type levels of μ-mRNA (i.e., *igm*933). It was suggested that genetic polarity in *E. coli* may result from degradation of mRNA prematurely exposed to attack by exo- and endoribonucleases (Morse and Yanofsky, 1969). Ribosomes appear to protect mRNA from degradation. As soon as a stop codon due to frameshift mutation is encountered ribosomes are detached, thus rendering the untranslatable mRNA hyperlabile (Hiraga and Yanofsky, 1972). If the rates of μ-mRNA synthesis in mutant (and wild-type) cells were similar, the mutants characterized here could be used to study various effects on mRNA stability, such as the influence of translated *versus* untranslated segments, directionality, and segmental specificity. In contrast to the other mutants, μ-mRNA in *igm*8279 is expressed at a rather high level although the mutation is presumably in the first half of Cμ3. Other mechanisms may account for this – either higher production or higher stability of its μ-mRNA. We are currently analysing the nature of its mutation.

Materials and methods

Cells and mutant selection

Cells were maintained and mutants selected as previously described (Köhler and Shulman, 1980). Each selection was performed on a single subclone of Sp6 (names in Table I) to ensure independence. Nine independently selected populations (Table I, see also Results) were screened for non-production of μ or κ chains. A solid phase radioimmunoassay for anti-idiotype (20-5) binding was performed using cell lysates. The antibody was obtained from P.A.Cazenave (Institut Pasteur, Paris). Cells from the selected populations were cloned under limiting dilution conditions, and half of a 0.2 ml culture was transferred to the wells of PVC plates coated with wild-type Sp6 IgM. The plates were centrifuged, the supernatants discarded, and 0.1 ml 1% Nonidet P-40 (NP-40) in phosphate buffered saline (PBS) was

added to lyse cells. To this mixture a limiting amount of [^{3}H]leucine-labeled anti-idiotypic antibody was added. The parental cell line, Sp6, served as positive control and *igm*10, a light chain only producing mutant daughter of Sp6, served as a negative control. DNA of cell lysates not inhibiting 20-5 binding to Sp6 IgM were further studied by Southern blot analysis. Clones not containing DNA sequences hybridizing to J_H or Cμ were considered as chromosome loss mutants and only clones displaying unaltered J_H and Cμ DNA fragments were further analyzed.

Screening with monoclonal antibodies

Rat monoclonal antibodies against each of the four mouse μ constant region domains were used. Antibodies against distinct non-overlapping determinants of single Cμ-domains were included (Leptin *et al.*, 1984). The mouse anti-idiotypic antibody 20-5 (IgG,κ) was purified over a protein A-Sepharose column and directly iodinated with ^{125}I (100 mCi/ml, Amersham, UK) to a specific activity of 500 μCi/10 μg protein. This reagent was at least 10 times more sensitive than the tritiated one used for the initial screening. All other antibodies were labeled by incubating 2 x 10^6 washed cells overnight in 2 ml of leucine-free medium containing 0.4 mCi [^{3}H]leucine (Amersham, UK, 120–190 Ci/mmol). After dialysis, 1–5 μl of the supernatants were used in radioimmunoassays. Affinity purified rabbit anti-mouse μ antibodies were used to coat 96 well polyvinyl chloride plates for 2 h at room temperature. Additional protein binding sites were blocked with a PBS/1% BSA solution (2 h, room temperature). Mutant and wild-type cell lysates were prepared by spinning down 2–9 x 10^6 cells and resuspending them in 500 μl PBS, with subsequent five rounds of freeze-thawing. Nuclei were spun out and 50 μl duplicates of the supernatants were incubated in the anti-μ coated wells. Bound material was revealed with the ^{125}I-labeled anti-idiotype or with each of the [^{3}H]leucine-labeled rat anti-μ monoclonal antibodies by counting the whole excised well in a scintillation counter. Values three times above background were considered positive in Table II. Plus signs not followed by the actual counts indicate independent determinations with different batches of labeled antibodies. The ^{125}I-labeled 20-5 antibody was used with a different batch of lysates so that here direct comparison of relative binding is not possible.

Ig analysis

To obtain unglycosylated protein chains, 10^7 cells were centrifuged, resuspended in DMEM containing 10 μg/ml tunicamycin (Sigma, St. Louis, MO) and grown for 30 min at 37°C. After a second centrifugation, cells were taken up in 2 ml leucine-free DMEM containing [^{14}C]leucine 100 μCi/ml (Amersham, UK; 130 Ci/mmol) and 10 μg/ml tunicamycin and grown for another 8 h. Cells were lysed in 0.8% NP-40, and nuclei removed by centrifugation. Igs in cell extract and supernatant were precipitated using a rabbit anti-mouse Ig serum and protein A-Sepharose (Pharmacia, Uppsala, Sweden). The precipitates were analysed by SDS-PAGE under reducing conditions.

For isoelectric focusing (IEF), cells were labeled as described above, omitting tunicamycin treatment. Cells were then removed by centrifugation and Igs in the supernatant reduced in the presence of 8 M urea, 1.5 M β-mercaptoethanol and 0.1 M potassium phosphate pH 8.0. The samples were then applied to polyacrylamide slabs containing ampholytes of the pH range 5–9 and 6 M urea (Köhler *et al.*, 1976).

Cloning of mutant μ-genes and DNA sequencing

DNA from the mutants *igm*145 and *igm*482 was digested with restriction enzyme *Eco*RI (Boehringer Mannheim). The fragmented DNA was separated by sucrose gradient centrifugation, fractions including the 13-kb fragments which contained the μ-gene were ligated to purified arms of the vector λgtwes λB for *igm*145, and λCh4A for *igm*482, respectively, *in vitro* packaged (Hohn, 1979) and plated on *E. coli* K803. The plaques containing the μ-gene bearing phage were detected as described (Benton and Davis, 1977) using a Cμ-cDNA probe pABμ-1 (Alt *et al.*, 1980). DNA of the purified phages was extracted, digested with *Pst*I and subcloned into M13 mp8 phage (Messing, 1981). DNA sequence analysis was performed according to Sanger *et al.* (1977).

RNA preparations

Cytoplasmic RNA was extracted from 2–6 x 10^8 cells using a modified method of Favaloro *et al.* (1980). Briefly, cells were lysed in 0.5% NP-40 and nuclei removed by centrifugation. After digestion with proteinase K and two extractions with phenol/chloroform/isoamylalcohol (25:24:1) RNA was used directly, or further fractionated on oligo(dT)-cellulose to obtain poly(A)$^+$ RNA. Agarose gel electrophoresis and transfer to nitrocellulose paper was done essentially as described (Thomas, 1980).

RNA dot blots

Dot blotting was performed as described (White and Bancroft, 1982) with the following modifications: to 30 μg of unfractionated cytoplasmic RNA from the various mutants, in a volume not exceeding 50 λ, 30 λ of formaldehyde (37% Merck) was added and the volume adjusted to 150 λ with 20 x SSC (3 M NaCl, 0.3 M Na citrate). The mixture was heated to 65°C for 5 min. In a 96 well costar tray containing 100 λ 20 x SSC per well, serial dilutions of 1:3 were performed. Then the various samples were transferred into a Schleicher and Schüll minifold dot blot apparatus containing a nitrocellulose blotting paper (Schleicher and Schüll GB003). Under vacuum the liquid was sucked through and RNA blotted to the filter. After the blots were dried under a lamp and baked *in vacuo* at 80°C for 2 h, hybridizations were performed as described (Southern, 1975).

RNA sequencing

The method used for RNA sequencing is an adaptation of the dideoxy sequencing procedure (Sanger *et al.*, 1977). To 3–5 μg of poly(A)$^+$ RNA 100 ng of the 17 base primer (starting in Cμ4 at amino acid position 454; synthesized by W.Bannwarth at Hoffmann-La Roche, Basel) and 9.75 μM dATP containing 20 μCi of 2000–3000 Ci/mmol [α-^{32}P]dATP was added. Salt conditions were adjusted to 100 mM Tris-HCl pH 8.3, 140 mM KCl, 10 mM $MgCl_2$, 20 mM β-mercaptoethanol. 13 units of AMV reverse transcriptase (RTase) (C.B.L. P.H.Stehelin) were added. The mixture, final volume 6 μl, was divided into four parts of 1.4 μl and 0.5 μl of the appropriate dideoxy-deoxy mixtures were added at final concentrations of 200 μM dGTP, dCTP, dTTP and 30 μM of the appropriate dideoxy nucleotide (except for ddATP which had a final concentration of 1.5 μM). After incubation for 30 min at 42°C, 1 μl chase solution containing 1 U/μl RTase, 0.25 mM of each deoxy nucleotide and appropriate RTase salt conditions were added and incubated for another 25 min at 42°C, then the temperature was raised to 45°C for 5 min to help RTase to move over RNA secondary structures. RNA was then hydrolysed by adding 2 μl of 0.25 M NaOH containing 25 mM EDTA and incubating at 65°C for 15 min. Finally 4 μl of formamide dye was added, the samples were incubated at 96°C for 3 min and 2 μl applied to a 6% or 12% sequencing gel.

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